Use of Random Amplified Polymorphic DNA (RAPD) Analysis for the Identification of *Giardia intestinalis* Subtypes and Phylogenetic Tree Construction

JITKA ŠEDINOVÁ,[a] **JAROSLAV FLEGR,**[a] **PETER L. EY**[b] **and JAROSLAV KULDA**[a]

[a]*Department of Parasitology, Faculty of Science, Charles University, Prague, 128 44, Czech Republic, and*
[b]*Department of Molecular Biosciences, Adelaide University, Adelaide SA 5005, Australia*

ABSTRACT. A comparison of random amplified polymorphic DNA (RAPD) was used to investigate genetic polymorphisms among 25 isolates of *Giardia intestinalis* and to assess the utility of RAPD for subtype detection and genealogical analysis. Using data obtained for six human and 19 animal-derived isolates in polymerase chain reactions using 13 different primers, phylogenetic trees were constructed and bootstrap values computed by the program FreeTree. Three major clades were distinguished, corresponding to previously defined genetic assemblages A, B, and E. The purported specificity of assemblage E genotypes for artiodactyl hosts was supported. Assemblages A and B showed wide host spectra, including human and animal hosts. No correlation was found between the genotype of analyzed isolates and the presence or absence of the double-stranded RNA Giardiavirus. The results indicate that RAPD data provide reliable genetic information that can be used for both ''fingerprinting'' and genealogical purposes.

Key Words. dsRNA virus, fingerprinting, genetic polymorphism, genotyping, polymerase chain reaction.

*G*IARDIA are ubiquitous gastrointestinal protozoan parasites that cause diarrhoeal disease in a wide range of host species. The taxonomy of the genus is based on morphological characters, particularly the shape and position of the median bodies, the shape of the cell, and the size of the ventral adhesive disc. Several morphological groups have been recognized and classified as species (Filice 1952; Kulda and Nohýnková 1996): *G. intestinalis* (Lambl, 1859) [syn. *G. duodenalis* (Davaine, 1875), and *G. lamblia* Stiles, 1915], *G. muris* (Grassi, 1879), *G. agilis* Künstler, 1882 (Feely and Erlandsen 1985) and *G. ardeae* Nöller, 1920 (Erlandsen et al. 1990b). However, some of these morphotypes appear to represent higher taxonomic units that include multiple species. Within the *G. intestinalis* group, two additional species, *G. psittaci* Erlandsen and Bemrick, 1987, and *G. microti* Kofoid and Christiansen, 1915 (Feely 1988), have been discerned on the basis of ultrastructural, developmental, and 18S rDNA data (van Keulen et al. 1998). Moreover, there is evidence of considerable genetic diversity among other members of this group.

Using phylogenetic analysis of nucleotide sequence and allozyme data, Monis et al. (1999) divided isolates of *G. intestinalis* into seven different lineages, designating assemblages A–G (review, Thompson et al. 2000). All characterized isolates from humans fall within two major genetic assemblages, A or B which also include many isolates from animals. Assemblages A and B each encompass a range of genotypes, with two distinct subgroups, Group I (subtype A-I) and Group II (subtype A-II), prominent within Assemblage A (Andrews et al. 1989; Ey et al. 1992–1997; Mayrhofer et al. 1995; Monis et al. 1996). Monis et al. (1996) have shown that assemblages A and B correspond respectively to the ''Polish'' and ''Belgian'' genotypes of Homan et al. (1992) and to groups [1 + 2] and 3 of Nash (Nash et al. 1985; Nash and Mowatt 1992). The other assemblages are defined by unique genotypes found only in animals: assemblages C and D (dogs; Monis et al. 1998), assemblage E (hoofed livestock; Ey et al. 1997), assemblage F (cats) and assemblage G (rats; Monis et al. 1999). The definition of genetic lineages that correlate with distinct phenotypic characters (Binz et al. 1992; Karanis and Ey 1998; Meloni and Thompson 1987; Monis et al. 1998; Weiss et al. 1992) supports the hypothesis that the *G. intestinalis* is a species complex consisting of several distinct species (Andrews et al. 1989). The restricted host range of isolates belonging to assemblages C-G contrasts with the broad host spectra observed for genotypes belonging to assemblages A and B. The A-I subtype represents a zoonotic genotype, as it is ubiquitous in human and animal hosts worldwide (Thompson 2000).

Techniques that have been used to compare *G. intestinalis* isolates genetically include analysis of allozymes (Andrews et al. 1989; Mayrhofer et al. 1995; Meloni et al. 1995; Strandèn et al. 1990), karyotypes (Sarafis and Isaac-Renton 1993; Upcroft et al. 1989), restriction fragment length polymorphisms (RFLP; Ey et al. 1992; Homan et al. 1992; Nash et al. 1985), nucleotide sequences (Baruch et al. 1996; Ey et al. 1993b, 1996, 1997; Monis et al. 1996, 1999; van Keulen et al. 1995, 1998; Weiss et al. 1992) and random amplified polymorphic DNA (RAPD; Morgan et al. 1993; Paintlia et al. 1999; van Belkum et al. 1993). RAPD is a DNA fingerprinting technique based on the amplification of undefined segments of the genome by PCR using single arbitrary primers (Williams et al. 1990). The multi-locus character of RAPD allows sensitive detection of polymorphisms in different parts of the genome. If performed with a sufficient number of primers, the method has the potential to provide reliable information about the genetic identity of isolates (Tibayrenc 1998). A drawback of the method is a requirement for DNA samples free of any contaminating DNA. Consequently, applicability of RAPD analysis is limited to axenic in vitro isolates which need not represent full spectrum of *G. intestinalis* diversity.

The aim of the present study was to evaluate the suitability of RAPD analysis for determining the genetic heterogeneity of *G. intestinalis* isolated mainly from animal sources and its utility for assessing the zoonotic potential of field isolates. Bootstrap analysis was used for the first time to estimate the robustness of a RAPD-based tree for *G. intestinalis*.

MATERIALS AND METHODS

***Giardia* isolates.** Data on the *G. intestinalis* isolates examined in this study are listed in Table 1. Assemblage A and B ''type'' references (Mayrhofer et al. 1995) included human-derived axenic isolates Ad-1/c7 (A, Group I), Bris-136/c1 (A, Group II) and Ad-28 (B) (Andrews et al. 1989; Ey et al. 1993a), as well as sheep isolate CH-O3 (A, Group I/II-like; Ey et al. 1996; Strandèn et al. 1990). The axenic Czech isolate P15, from a pig (Koudela et al. 1991), served as a type reference for genetic assemblage E (Ey et al. 1997; Monis et al. 1999).

Three other isolates were of human origin: HP-1 [= Portland 1 (Meyer 1976), ATCC 30888] obtained from Prof. A. E. Meyer (Oregon Health Science University, Portland, OR); HH, isolated in Prague from a Czech visitor to India; and Ber-1, isolated from a patient with treatment-refractory chronic giardiasis.

Corresponding Author: J. Kulda—Telephone number: 420 221 951 812; FAX number: 420 224 919 704; E-mail: kulda@natur.cuni.cz

Table 1. Isolates of *Giardia intestinalis* examined in this study.

Isolate code	Host origin	Geographic origin	Known genotype†	References
Ad-1/c7	Human	Australia, Adelaide	A-I	a,d,j
Ad-28	Human	Australia, Adelaide	B	d,h,j
Bris-136/c1	Human	Australia, Brisbane	A-II	a,h,j
HP-1*	Human	USA, Portland	A-I	i
Ber-1	Human	CZ**, Prague		
HH	Human	India		
CH-O3	Sheep	Switzerland	A-I/II	e,l
J 99/9	Sheep	CZ, Vsetín	E	f,k
J 17/10	Sheep	CZ, Orlov	E	f,k
J III	Sheep	CZ, Vsetín		
MUF-1	Mouflon	CZ, Zěhrov		
GN	Bearded argali	CZ, ZOO Prague		
P15	Pig	CZ, Cěské Budě-jovice	E	f,g
Kun	Horse	CZ, Chomutov		
Ko-1	Cat	CZ, Vsetín		
Ko-188	Cat	CZ, Prague		
D47	Dog	CZ, Prague	B	k
P117	Cuis	Poland, ZOO Poznan	B	b,j
Ch-46	Chinchilla	CZ, Prague		
Ch-Pa 911	Chinchilla	CZ, Kolín		
Ch-105	Chinchilla	CZ, Prague		
Beaver ORI	Beaver	USA		c
DIKO	Porcupine	CZ, ZOO Chomutov		
P114	Gambian giant pouched rat	Poland, ZOO Poznan	B	b,j
P2-MER	Pig	CZ, Cěské Budě-jovice	A-I	f

ᵃ Andrews et al. 1989, ᵇ De Jonckheere et al. 1990, ᶜ Erlandsen et al. 1990b, ᵈ Ey et al. 1993, ᵉ Ey et al. 1996, ᶠ Ey et al. 1997, ᵍ Koudela et al. 1991, ʰ Mayrhofer et al. 1995, ⁱ Meyer 1976, ʲ Monis et al. 1996, ᵏ Monis et al. 1998, ˡ Strandèn et al. 1990.

* Portland-1, American Type Culture Collection #30888.

** CZ = Czech Republic.

† A, B or E = assemblage A, B or E respectively; A-I = assemblage A, Group I (subtype A-I); A-II = assemblage A, Group II (subtype A-II); A-I/II = assemblage A, Group I/II-like.

Two rodent isolates, P 114 (= GG PRP-114 from a Gambian giant pouched rat, *Cricetomys gambianus*) and P 117 (= CP-117 from a South American cuis, *Galea musteloides*) established in Poland from animals maintained in the Poznan Zoological Garden (De Jonckheere et al. 1990), had been tested previously (Monis et al. 1996). A culture of beaver *Giardia* (Beaver ORI) provided by Prof. Meyer was originally established from fecal samples of kill-trapped beaver by Erlandsen et al. (1990b). Other isolates listed in Table 1 were established in axenic culture from domestic or captive animals in the Czech Republic. All these isolates are deposited in the culture collection of the Department of Parasitology, Charles University, Prague.

Cultivation in vitro. *Giardia intestinalis* isolates used for DNA isolation were initiated from cryopreserved samples maintained in liquid nitrogen. Cultures were grown axenically in TYI-S-33 medium modified according to Keister (1983) containing 10% heat-inactivated bovine serum, bovine bile, and antibiotics (penicillin 1,000 IU/ml, amikacin 250 μg/ml). Cultures were incubated at 37 °C in 7.5-ml screw-cap tubes in a

Table 2. Oligodeoxynucleotide primers used for RAPD analysis of *Giardia intestinalis* isolates and clones.

Primer code	Nucleotide sequence (5′ → 3′)	Analysis of isolates	Analysis of clones
UBC 151	GCT GTA GTG T	×	×
UBC 155	CTG GCG GCT G	×	×
UBC 156	GCC TGG TTG C		×
UBC 159	GAG CCG GTA G	×	×
UBC 164	CCA AGA TGC T	×	×
UBC 168	CTA GAT CTG C	×	×
UBC 172	ACC GTC GTA G		×
UBC 179	TCA CTG TAC G	×	
UBC 180	GGG CCA CGC T	×	×
UBC 181	ATG ACG ACG G	×	×
UBC 182	GTT CTC GTG T	×	
UBC 189	TGC TAG CCT C	×	×
UBC 192	GCA AGT CAC T		×
UBC 194	AGG ACG TGC C		×
UBC 195	GAT CTC AGC G	×	×
UBC 198	GCA GGA CTG C	×	×
UBC 199	GCT CCC CCA C	×	
UBC 200	TCG GGA TAT G		×

horizontal position and transferred (or harvested) after the trophozoites had formed an adherent monolayer (4–8 days).

Clonal lines. Clonal lines of isolate P15 were established from micro-drops of a cell suspension that were placed on fragments of sterilized coverslips and determined by microscopy to contain a single trophozoites (Binz et al. 1991).

DNA isolation. Adherent trophozoites were detached from the tubes in cold phosphate-buffered saline, pH 7.4 (PBS) and after chilling the tubes on ice for 15 min, the cells were harvested by centrifugation (1,000 *g,* 10 min). The cell pellet was resuspended in 200 μl of lysis buffer (0.5% Sarkosyl/0.15 M NaCl/50 mM Tris-HCl/0.5 mM EDTA, pH 8.0) and shaken for 2 min. The lysate was extracted twice, first with phenol/chloroform/isoamylalcohol (25:24:1), then with chloroform/isoamylalcohol (24:1). DNA was precipitated at −20 °C (overnight) by addition of 2.5 vol. of 96% ethanol. The pellet was washed in 70% ethanol, air-dried and dissolved in 100 μl of sterile distilled water. After treatment with RNase A (Sigma, St. Louis, MO; 20 μg/ml, 30 min, 36 °C), the phenol extraction and ethanol precipitation were repeated. The DNA pellet was dissolved in 50 μl of sterile distilled water and stored at −20 °C.

Detection of dsRNA virus. For detection of the double-stranded RNA (dsRNA) Giardiavirus, total nucleic acid extracts (not treated with RNAse A) were subjected to electrophoresis in 1% agarose gels. The dsRNA was recognized as a distinct satellite band (below the wide band of genomic DNA) with a mobility corresponding to a 7.0-kb DNA molecule.

RAPD analysis. Polymerase chain reactions (PCR) were performed in 20-μl reaction vol. containing: PCR buffer without MgCl₂ (0.1% Triton X-100, 50 mM KCl, 10 mM Tris-HCl, pH 9.0; Promega Corporation, Madison, WI), 2.5 mM MgCl₂ (Promega), 200 μM of each dNTP (Promega), 10 μM of a single oligodeoxynucleotide primer (University of British Columbia, NC-PS Unit, BC), 2 units of *Taq* DNA polymerase (Promega), and 10 ng of template DNA. In preliminary analyses, 30 random primers were tested with DNA samples of one *G. intestinalis* isolate (HP-1) and only those, that produced rich banding patterns were chosen for testing other isolates. Samples from the 25 *G. intestinalis* isolates were analyzed by RAPD using 13 primers, whereas clones derived from isolate P15 were analyzed using 15 primers (Table 2). PCR were performed using a PT C-200 thermocycler (MJ Research, Inc., Watertown,
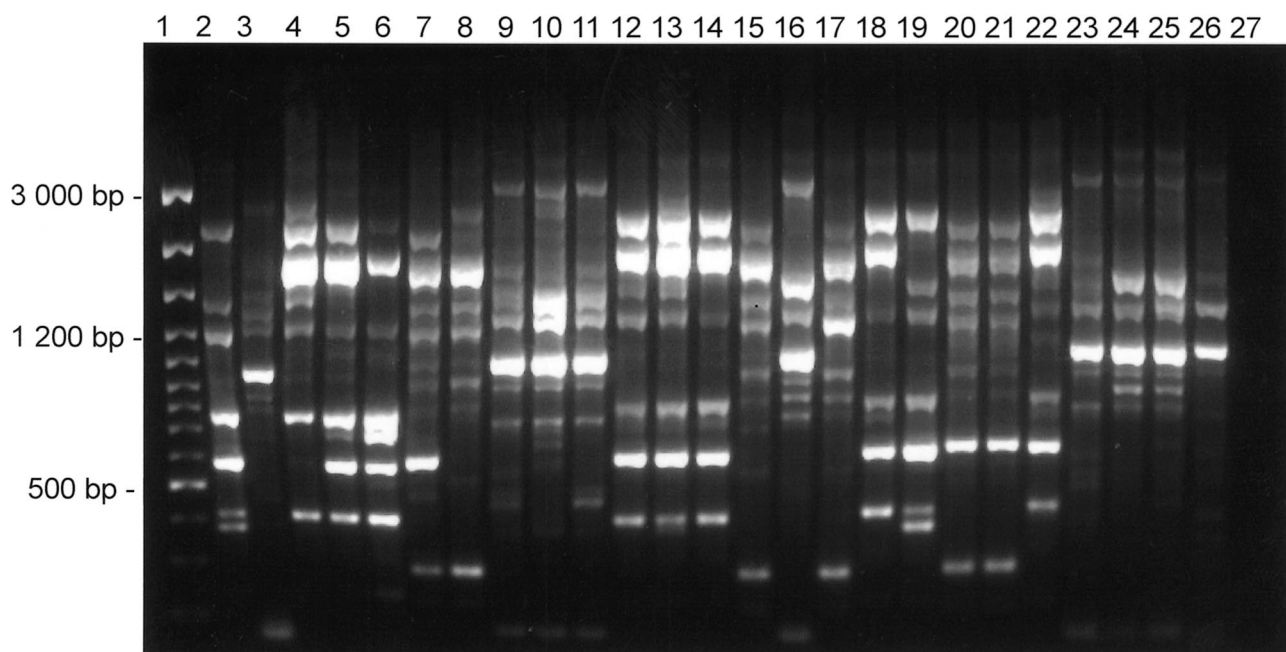
Fig. 1. Results of PCR-fingerprinting using RAPD electrophoretic patterns obtained for 25 isolates of *Giardia intestinalis* using primer UBC 180. Lanes: **2**- Bris 136/c1 (human), **3**- Ad-28 (human), **4**- CH-O3 (sheep), **5**- Ad-1/c7 (human), **6**- HP-1 (human), **7**- J 17/10 (sheep), **8**- P15 (pig), **9**- Ch-105 (chinchilla), **10**- Ch-Pa 911 (chinchilla), **11**- D47 (dog), **12**- KUN (horse), **13**- Ko-188 (cat), **14**- Beaver ORI (beaver), **15**- Muf-1 (mouflon), **16**- Diko (porcupine), **17**- GN (bearded argali), **18**- P2-MER (pig), **19**- Ber-1 (human), **20**- J99/9 (sheep), **21**- J III (sheep), **22**- Ko-1 (cat), **23**- Ch-46 (chinchilla), **24**- P117 (cuis), **25**- P114 (Gambian giant pouched rat), **26**- HH (human), **27**- Negative control (no template DNA). Lane 1 contained size standards ("100bp DNA Ladder Plus", MBI Fermentas, Vilnius, Lithuania).

MA) programmed for an initial denaturation step at 92 °C (100 s) followed by 35 reaction cycles (94 °C/40 s, 32 °C/40 s, 72 °C/90 s) and a final extension step at 72 °C (10 min). RAPD products were identified after electrophoresis in 2% agarose gels (TAE buffer) and staining with ethidium bromide. For each primer, DNA samples from all 25 isolates (or the 14 clones) were tested simultaneously in a single reaction run. The complete reaction mixture (minus template DNA) was prepared as a single batch and aliquots were dispensed into 0.2-ml PCR tubes containing test DNA samples or water (negative control).

Data analysis. The electrophoretograms were captured by digital camera (model ST-7) using PC program SkyPro. The gels were displayed on a computer screen and the bands obtained were transformed manually into a binary data matrix, i.e. individual bands obtained using a given primer were scored as present (1) or absent (0) for each isolate. All visible electrophoretic bands were included in the RAPD analysis. Genetic distances [d] of samples were computed from Nei-Li's coefficient of similarity [s] (Nei and Li 1979), where d = $(1 - s)$. Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei 1987) as well as by the unweighted pair-grouping method (UPGMA) (Sneath and Sokal 1973). The robustness of trees was assessed by bootstrap analysis (250 replications). Computations of genetic distances, construction of trees, and bootstrap analyses were performed by the program FreeTree (Hampl et al. 2001; Pavlíček et al. 1999).

Statistical testing of tree concordance. Permutation tail probability testing was used to estimate the correspondence of the presence of dsRNA virus (Giardiavirus) in examined isolates with their position in the tree (Vaňáčová et al. 1997). The presence of Giardiavirus was coded as 1 and its absence as 0. The test was done with the program Treept (Flegr et al. 1998).

RESULTS

RAPD analysis of *Giardia intestinalis* strains. Samples of DNA from 25 *G. intestinalis* isolates, including "type" references for genetic assemblages A (A-I, A-II, A-I/II), B and E, were tested in single-primer PCR using 13 different oligonucleotides (Table 2). Each primer yielded numerous amplified DNA fragments, as exemplified in Fig. 1 for primer UBC 180. The overall banding patterns differed markedly, a unique banding pattern being obtained for each "type" isolate (Fig. 1). Comparisons with the test isolates indicated that the banding patterns were related to the genotype of the organisms, the differences between isolates that yielded a similar overall banding profile being, in general, only minor (c.f. lanes 9–11, 16 and 23–25). Similar results were obtained using the other primers, the banding patterns being largely distinctive for one or other of the three defined genetic assemblages (data not shown).

In order to compare the banding data rigorously, all of the bands evident in each data (primer) set were allotted binary status (1 = present, 0 = absent) and used to build a data matrix. The final matrix containing 407 binary RAPD characters (bands) from all 25 isolates was used for construction of phylogenetic trees (Fig. 2). Both NJ (Fig. 2) and UPKMA methods (data not shown) produced similar results. The isolates formed three distinct clades, supported by high bootstrap values ($\geq$ 99%). The distribution of "type" isolates within the tree indicated that these clades represented the three genetic assemblages A, B, and E defined by Monis et al. (1999). The mean interclade Nei-Li distances were similar (A:B = 0.75, A:E = 0.76, B:E = 0.72). These were approximately twice the mean intraclade distances (0.36, 0.39, and 0.25 for assemblage A, B, and E respectively), consistent with the high bootstrap support for each clade.
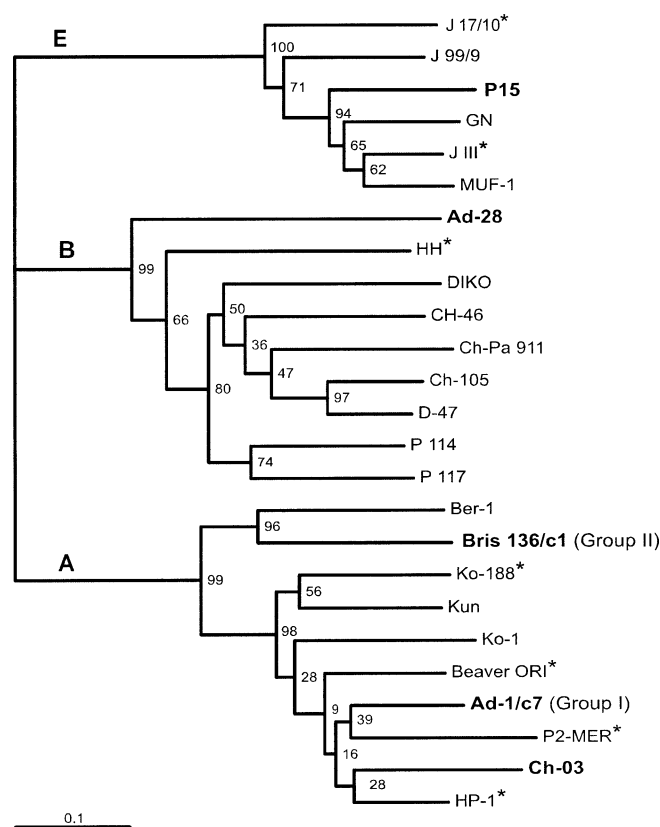
Fig. **2.** Phylogenetic tree for 25 isolates of *Giardia intestinalis* constructed from pooled transformed RAPD data by the Neighbour-Joining method and separated in Assemblages A, B, and E. Branch lengths reflect relative genetic distances between isolates. Bootstrap values for particular branches are indicated. Isolate names printed in bold designate type references for particular genetic assemblages. Asterisks designate isolates in which Giardiavirus dsRNA was detected.

The assemblage A clade comprised ten isolates: three ''type'' references (Ad-1/c7, Bris-136/c1 from humans, and CH-O3 from sheep), together with two human (HP-1, Ber-1) and five animal isolates from cats, horse, pig, and beaver. Within this assemblage, the Group I and Group II ''type'' isolates were resolved within separate clades, with bootstrap values of 98% and 96% respectively (Fig. 2). Seven isolates co-clustered with the Group I reference (Ad-1/c7), but none showed identity with this isolate. Bootstrap support for the branching order within this clade was extremely weak (mean, 38.3%). Only one isolate, Ber-1 (human-derived), co-clustered with the Group II reference, Bris-136/c1, but the two isolates were not identical.

The assemblage B clade included two human isolates, HH and the Ad-28 ''type'' reference, plus seven animal isolates—from dog, chinchillas, porcupine, and exotic rodents (*C. gambianus*, *G. musteloides*) (Fig. 2). The internal branching order was not well supported (mean bootstrap value, 65%).

The third major clade, corresponding to assemblage E (comprising ''Hoofed livestock'' genotypes), contained six isolates—all from hosts within the Order Artiodactyla (pig, sheep, bearded argali, mouflon). Clonal lines derived from isolate P15 were genetically homogenous and stable (data not shown).

Giardiavirus detection. Giardiavirus dsRNA was detected in the nucleic acid samples from seven of 21 isolates examined (Fig. 2). These included representatives of assemblage A (four isolates), assemblage B (one isolate), and assemblage E (two isolates). No statistically significant concordance was detected

between the presence or absence of Giardiavirus RNA and the position of isolates within the phylogenetic tree (p = 0.152).

DISCUSSION

The findings of this study show that RAPD can be used to reliably identify distinct subtypes of *G. intestinalis* and that banding data, if transformed to binary form for incorporation into data matrices, are suitable for phylogenetic reconstructions. Our analysis of 25 isolates distinguished three highly distinct clades, corresponding to genetic assemblages A, B, and E, which had been defined previously on the basis of nucleotide sequence and other data (Ey et al. 1997; Mayrhofer et al. 1995; Monis et al. 1996, 1999; Thompson et al. 2000). The three clades representing assemblages A, B and E were supported by very high bootstrap values (≥ 99%). All of the ''type'' isolates were placed correctly into separate clades or, for the multiple assemblage A ''type'' samples, into the same major clade. The distinctive, stable RAPD banding patterns observed in this study contrast with previous RAPD data reported for *G. intestinalis*. However, the isolates examined in these earlier studies were either less diverse genetically than those comprising the current panel (e.g. Le Blanc et al. 1991; Paintlia et al. 1999), or else the patterns were interpreted as ''fingerprints'' (with definition of 10 ''rapdemes''; Morgan et al. 1993) or as evidence of genetic ''variation'' among isolates or clones. These differences can be attributed in part to high frequency chromosome rearrangements occurring within telomeric repeats and rDNA tandem arrays (Le Blanc et al. 1991; van Belkum et al. 1993). Such alterations in chromosome structure do not reflect long-term (mutational) evolutionary changes and they are not observed in genetic assays that detect nucleotide substitutions within protein-encoding or rRNA genes. Similarly, they would not be detected by RAPD analysis, unless the RAPD primers targeted telomeric segments that span chromosomal breakpoints.

The differentiation of isolates within each assemblage by RAPD analysis has a less certain genetic basis than the resolution of the three major clades, as the distinctions rest on more subtle, undefined differences. Nevertheless, the resolution of Group I, Group II, and Group I/II-like ''type'' isolates, which are known to be genetically distinct members of assemblage A (Monis et al. 1996), indicates that the differentiation observed between all of the isolates is likely to be correct, even if the branching order is uncertain. In this context, use of conventional assays (Karanis and Ey 1998) to type several of these isolates on the basis of RFLP indicated that isolate Ber-1 was identical to Bris-136/c1 and that isolates P2-MER, Ko-1, and Ko-188 were identical to Ad-1/c7. On this basis, RAPD analysis is more discriminatory but the significance of the fine differences observed by this technique between nominally identical isolates is unclear. In terms of biological significance, the detection of Giardiavirus dsRNA in isolates belonging to genetic assemblages A, B, and C suggests that susceptibility to viral infection is not genotype-dependent.

The recovery of Group I (subtype A-I) organisms and closely related genotypes from many different animals (e.g. cats, dogs, beavers, cattle, horses, and sheep) as well as humans, as demonstrated in this and previous studies (Ey et al. 1996; Homan et al. 1992; Karanis and Ey 1998; Monis et al. 1996; Nash and Mowatt 1992), indicates that the A-I subtype has a broad host spectrum and high transmissibility and that it thus represents a threat of zoonotic infection (Thompson et al. 1990). Members of this group have been identified by different analytical techniques worldwide and interpreted as descendants of a successful clone that has become dispersed in relatively recent time by human migration (Monis et al. 1996). Group II isolates (subtype

A-II) have so far been recovered almost exclusively from humans (Karanis and Ey, 1998; Ponce-Macetola et al. 2002).

The clade identified by RAPD as assemblage B consisted of two human isolates (Ad-28 and HH), one dog isolate (D47), three isolates from chinchillas and others from animals in zoos—a porcupine and two exotic rodents, a cuis, and a Gambian giant pouched rat. One assemblage B isolate (P114, derived from a giant pouched rat) caused giardiasis in an experimentally infected human volunteer (Majewska 1994). Our axenic *G. intestinalis* isolate D47 from a dog, was shown previously to belong to assemblage B (Monis et al. 1998), unlike Australian canine isolates (established by propagation in suckling mice), which were found to be specific ''dog'' genotypes that define assemblages C and D (Monis et al. 1998). The low success rate in establishing *Giardia* from dogs in vitro is a notorious problem (Kulda and Nohýnková 1996), probably due to differences in the metabolic capacity of assemblage C and D subtypes.

Six isolates, including the reference strain from pig (P-15), formed the clade recognized as genetic assemblage E. This assemblage has been found, in this study and previously (Ey et al. 1997; Thompson et al. 2000), to consist only of *Giardia* recovered from the mammalian Order Artiodactyla. In the present study using RAPD, isolates derived from a pig, sheep, a bearded argali, and a mouflon formed the assemblage E clade. These genotypes defined originally by Ey et al. (1997) seem to have a narrow host spectrum confined to cloven-hoofed animals.

In conclusion, our results confirm the utility of RAPD analysis for genealogical studies with axenic isolates of *G. intestinalis* and possibly other organisms. The multi-locus character of RAPD, the capacity to undertake phylogenetic reconstructions on the basis of binary transformed RAPD banding data, and the means to test the resulting phylogenetic trees by bootstrap analysis make this technique a valuable supplement to other methods used in epidemiological studies.

ACKNOWLEDGMENTS

LITERATURE CITED

Andrews, R. H., Adams, M., Boreham, P. F., Mayrhofer, G. & Meloni, B. P. 1989. *Giardia intestinalis*: electrophoretic evidence for a species complex. *Int. J. Parasitol.,* **19**:183–190.

Baruch, A. C., Isaac-Renton, J. L. & Adam, R. D. 1996. The molecular epidemiology of *Giardia lamblia*: a sequence-based approach. *J. Infect. Dis.,* **174**:233–236.

Binz, N., Thompson, R. C. A., Lymbery, A. J. & Hobbs, R. P. 1992. Comparative studies on the growth dynamics of two genetically distinct isolates of *Giardia duodenalis* in vitro. *Int. J. Parasitol.,* **22**:195–202.

Binz, N., Thompson, R. C. A., Meloni, B. P. & Lymbery, A. J. 1991. A simple method for cloning *Giardia duodenalis* from cultures and faecal samples. *J. Parasitol.,* **77**:627–631.

De Jonckheere, J. F. & Gordts, B. 1987. Occurrence and transfection of a *Giardia* virus. *Mol. Biochem. Parasitol.,* **23**:85–89.

De Jonckheere, J. F., Majewska, A. C. & Kasprzak, W. 1990. *Giardia* isolates from primates and rodents display the same molecular polymorphism as human isolates. *Mol. Biochem. Parasitol.,* **39**:23–29.

Erlandsen, S. L. & Bemrick, W. J. 1987. SEM evidence for a new species, *Giardia psittaci. J. Parasitol.,* **73**:623–629.

Erlandsen, S. L., Sherlock, L. A., Bemric, W. J., Ghobrial, H. & Jakubowski, W. 1990b. Prevalence of *Giardia* spp. in beaver and muskrat population in northeastern states and Minnesota: detection of intestinal trophozoites at necropsy provides greater sensitivity than detection of cysts in faecal samples. *Appl. Environ. Microbiol.,* **56**:31–36.

Erlandsen, S. L., Bemrick, W. J., Wells, C. L., Feely, D. E., Knudson, L., Campbell, S. R., van Keulen, H. & Jarroll, E. L. 1990a. Axenic culture and characterization of *Giardia ardeae* from the great blue heron (*Ardea herodias*). *J. Parasitol.,* **76**:717–724.

Ey, P. L., Andrews, R. H. & Mayrhofer, G. 1993. Differentiation of major genotypes of *Giardia intestinalis* by polymerase chain reaction analysis of a gene encoding a trophozoite surface antigen. *Parasitology,* **106**:347–356.

Ey, P. L., Bruderer, T., Wehrli, C. & Köhler, P. 1996. Comparison of genetic groups determined by molecular and immunological analyses of *Giardia* isolated from animals and humans in Switzerland and Australia. *Parasitol. Res.,* **82**:52–60.

Ey, P. L., Darby, J. M., Andrews, R. H. & Mayrhofer, G. 1993. *Giardia intestinalis*: detection of major genotypes by restriction analysis of gene amplification products. *Int. J. Parasitol.,* **23**:591–600.

Ey, P. L., Khanna, K., Andrews, R. H., Manning, P. A. & Mayrhofer, G. 1992. Distinct genetic groups of *Giardia intestinalis* distinguished by restriction fragment length polymorphisms. *J. Gen. Microbiol.,* **138**:2629–2637.

Ey, P. L., Mansouri, M., Kulda, J., Nohýnková, E., Monis, P. T., Andrews, R. H. & Mayrhofer, G. 1997. Genetic analysis of *Giardia* from hoofed farm animals reveals artiodactyl-specific and potentially zoonotic genotypes. *J. Eukaryot. Microbiol.,* **44**:626–635.

Feely, D. E. 1988. Morphology of the cyst of *Giardia microti* by light and electron microscopy. *J. Protozool.,* **35**:52–54.

Feely, D. E. & Erlandsen, S. L. 1985. Morphology of *Giardia agilis*: observation by scanning electron microscopy and interference reflexion microscopy. *J. Protozool.,* **32**:691–693.

Filice, F. P. 1952. Studies on the cytology and life history of a *Giardia* from the laboratory rat. *Univ. Calif. Publ. Zool.,* **57**:53–143.

Flegr, J., Záboj, P. & Vaňáčová, Š. 1998. Correlation between aerobic and anaerobic resistance to metronidazole in trichomonads: application of a new computer program for permutation tests. *Parasitol. Res.,* **84**:590–592.

Hampl, V., Pavlíček, A. & Flegr, J. 2001. Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with the freeware program FreeTree: application to trichomonad parasites. *Int. J. System. Evol. Microbiol.,* **51**:731–735.

Homan, W. L., van Enckevort, F. H. J., Limper, L., van Eys, G. J. J. M., Schoone, G. J., Kasprzak, W., Majewska, A. C. & van Knapen, F. 1992. Comparison of *Giardia* isolates from different laboratories by isoenzyme analysis and recombinant DNA probes. *Parasitol. Res.,* **78**:316–323.

Karanis, P. & Ey, P. L. 1998. Characterization of axenic isolates of *Giardia intestinalis* established from humans and animals in Germany. *Parasitol. Res.,* **84**:442–449.

Keister, D. B. 1983. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Trans. R. Soc. Trop. Med. Hyg.,* **77**:487–488.

Koudela, B., Nohýnková, E., Vítovec, J., Pakandl, M. & Kulda, J. 1991. *Giardia* infection in pigs: detection and in vitro isolation of trophozoites of the *Giardia intestinalis* group. *Parasitology,* **102**:163–166.

Kulda, J. & Nohýnková, E. 1996. *Giardia* in humans and animals. *In*: Kreier, J. P (ed.), Parasitic Protozoa, 2nd ed., Academic Press, San Diego. **10**:225–422.

Le Blancq, S. M., Korman, S. H. & Van der Ploeg, L. H. T. 1991. Frequent rearrangements of rRNA-encoding chromosomes in *Giardia lamblia. Nucl. Acids Res.,* **19**:4405–4412.

Majewska, A. C. 1994. Successful experimental infections of a human volunteer and Mongolian gerbils with *Giardia* of animal origin. *Trans. R. Soc. Trop. Med. Hyg.,* **88**:360–362.

Mayrhofer, G., Andrews, R. H., Ey, P. L. & Chilton, N. B. 1995. Division of *Giardia* isolates from humans into two genetically distinct assemblages by electrophoretic analysis of enzymes encoded at 27 loci and comparison with *Giardia muris. Parasitology,* **111**:11–17.

Meloni, B. P. & Thompson, R. C. A. 1987. Comparative studies on the axenic in vitro cultivation of *Giardia* of human and canine origin: evidence for intra-specific variation. *Trans. R. Soc. Trop. Med. Hyg.,* **81**:637–640.

Meloni, B. P., Lymbery, A. J. & Thompson, R. C. A. 1995. Genetic characterization of isolates of *Giardia duodenalis* by enzyme electrophoresis: implications for reproductive biology, population structure, taxonomy, and epidemiology. *J. Parasitol.,* **81**:368–383.

Meyer, E. A. 1976. *Giardia lamblia*: isolation and axenic cultivation. *Exp. Parasitol.,* **39**:101–105.

Monis, P. T., Andrews, R. H., Mayrhofer, G. & Ey, P. L. 1999. Molecular systematics of the parasitic protozoan *Giardia intestinalis*. *Mol. Biol. Evol.,* **16**:1135–1144.

Monis, P. T., Mayrhofer, G., Andrews, R. H., Homan, W. L., Limper, L. & Ey, P. L. 1996. Molecular genetic analysis of *Giardia intestinalis* isolates at the glutamate dehydrogenase locus. *Parasitology,* **112**: 1–12.

Monis, P. T., Andrews, R. H., Mayrhofer, G., Mackrill, J., Kulda, J., Isaac-Renton, J. L. & Ey, P. L. 1998. Novel lineages of *Giardia intestinalis* identified by genetic analysis of organisms isolated from dogs in Australia. *Parasitology,* **116**:7–19.

Morgan, U. M., Constantine, C. C., Greene, W. K. & Thompson, R. C. A. 1993. RAPD (random amplified polymorphic DNA) analysis of *Giardia* DNA and correlation with isoenzyme data. *Trans. R. Soc. Trop. Med. Hyg.,* **87**:702–705.

Nash, T. E. & Mowatt, M. R. 1992. Identification and characterization of a *Giardia lamblia* group-specific gene. *Exp. Parasitol.,* **75**:369–378.

Nash, T. E., McCutchan, T., Keister, D., Dame, J. B., Conrad, J. D. & Gillin, F. D. 1985. Restriction-endonuclease analysis of DNA from 15 *Giardia* isolates obtained from humans and animals. *J. Infect. Dis.,* **152**:64–73.

Nei, M. & Li, W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA,* **76**:5269–5273.

Paintlia, A. S., Mahajan, R. C., Chakraborti, A., Sehgal, R. & Ganguly, N. K. 1999. Characterization of *Giardia lamblia* groups A and B from North India by isoenzyme and random amplified polymorphic DNA analysis. *Parasitol. Res.,* **85**:510–512.

Pavlíček, A., Hrdá, Š. & Flegr, J. 1999. FreeTree freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of genus *Frenkelia. Folia Biol. (Praha),* **45**:97–99.

Ponce-Macotela, M., Martínez-Gordillo, M. N., Bermüdez-Cruz, R. M., Salazar-Schettino, P. M., Ortega-Pierres, G. & Ey, P. L. 2002. Unusual prevalence of the *Giardia intestinalis* A-II subtype amongst isolates from humans and domestic animals in Mexico. *Int. J. Parasitol.,* **32**:1201–1202.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* **4**:406–425.

Sarafis, K. & Isaac-Renton, J. L. 1993. Pulsed-field gel electrophoresis as a method of biotyping of *Giardia duodenalis. Am. J. Trop. Med. Hyg.,* **48**:134–144.

Sneath, P. H. & Sokal, R. R. 1973. Numerical Taxomomy. W. H. Freeman, San Francisco, CA.

Strandèn, A. M., Eckert, J. & Köhler, P. 1990. Electrophoretic characterization of *Giardia* isolated from humans, cattle, sheep, and a dog in Switzerland. *J. Parasitol.,* **76**:660–668.

Thompson, R. C. A. 2000. Giardiasis as a re-emerging infectious disease and its zoonotic potential. *Inter. J. Parasitol.,* **30**:1259–1267.

Thompson, R. C. A., Hopkins, R. M. & Homan, W. L. 2000. Nomenclature and genetic groupings of *Giardia* infecting mammals. *Parasitol. Today,* **16**:210–213.

Thompson, R. C. A., Lymbery, A. J., Meloni, B. P. & Binz, N. 1990. The zoonotic transmission of *Giardia* species. *Vet. Rec.,* **126**:513–514.

Tibayrenc, M. 1998. Genetic epidemiology of parasitic protozoa and other infectious agents: the need for an integrated approach. *Int. J. Parasitol.,* **28**:85–104.

Upcroft, J. A., Boreham, P. F. & Upcroft, P. 1989. Geographic variation in *Giardia* karyotypes. *Int. J. Parasitol.,* **19**:519–527.

van Belkum, A., Homan, W., Limper, L. & Quint, W. G. 1993. Genotyping isolates and clones of *Giardia duodenalis* by polymerase chain reaction: implications for the detection of genetic variation among protozoan parasite species. *Mol. Biochem. Parasitol.,* **61**:69–77.

van Keulen, H., Homan, W. L., Erlandsen, S. L. & Jarroll, E. L. 1995. A three nucleotide signature sequence in small subunit rRNA divides human *Giardia* in two different genotypes. *J. Eukaryot. Microbiol.,* **42**:392–394.

van Keulen, H., Feely, D. E., Macechko, P. T., Jarroll, E. L. & Erlandsen, S. L. 1998. The sequence of *Giardia* small subunit rRNA shows that voles and muskrats are parasitized by a unique species *Giardia microti. J. Parasitol.,* **84**:294–300.

Vaňáčová, Š., Tachezy, J., Kulda, J. & Flegr, J. 1997. Characterization of trichomonad species and strains by PCR fingerprinting. *J. Eukaryot. Microbiol.,* **44**:545–552.

Weiss, J. B., van Keulen, H. & Nash, T. E. 1992. Classification of subgroups of *Giardia lamblia* based upon ribosomal RNA gene sequence using the polymerase chain reaction. *Mol. Biochem. Parasitol.,* **54**:73–86.

Williams, J. G. K., Kubelík, A. R., Livak, K. J., Rafalski, J. A. & Tingey S. V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.,* **18**:6531–6535.